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Anti-inflammatory Activity of *Ocimum americanum* L. Essential Oil in Experimental Model of Zymosan-Induced Arthritis

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Abstract: Essential oils are potential sources of novel components for medicinal use. The present study was performed to investigate the composition and anti-inflammatory activity of *Ocimum americanum* L. essential oil (OEO) and its components in an experimental model of zymosan-induced arthritis and paw edema. The essential oil was obtained by hydrodistillation and analyzed by gas chromatography-mass spectrometry. Twenty-six components, representing 98.9% of the total oil, were characterized, with linalool (19.63%) and 1,8-cineole (17.27%) as the main components. The OEO and its two constituents inhibited leukocyte influx into the synovial space and reduced paw edema induced by zymosan. The OEO also inhibited interferon-γ levels but did not reduce transforming growth factor-β levels. Additionally, the OEO protected against leukocyte influx into the synovial membrane and cartilage destruction in knee joints in arthritic mice. These findings indicate that the essential oil of *Ocimum americanum* L. exerted significant anti-inflammatory effects, likely related to its main compounds.

Keywords: *Ocimum americanum* L.; Essential Oil; Leukocyte Migration.

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Introduction

Essential oils are volatile substances that have been evolutionarily selected for the survival of plants within their ecosystems, and they play a key role in defense against microorganisms and predators (Bakkali *et al.*, 2008; Pino *et al.*, 2009). Chemically, essential oils mostly consist of terpenic substances and phenylpropanoids, in which the relative proportions of these components vary, depending on the geographic region and method of extraction (Sacchetti *et al.*, 2004). All plant species accumulate these volatile elements in specific anatomic organs (Hyldgaard *et al.*, 2012).

Among the families of plants that concentrate volatile substances in their leaves, the family Lamiaceae, genus *Ocimum* (basil), consists of more than thirty species. It is native to tropical and subtropical regions and has been cultivated as edible herbs in many countries. Its leaves are used as culinary condiments, and their essential oils have been used extensively in food and perfume products (Agostini *et al.*, 2009; Rady and Nazif, 2005).

With regard to folk medicine, many species in this family are used to treat insomnia, constipation, cough, and microbial infections (Yucharoen *et al.*, 2011). Studies have also reported the antioxidant (Lee *et al.*, 2005), hypocholesterolemic (Bravo *et al.*, 2008), and antifungal (Khan *et al.*, 2010) effects of the extracts and essential oils of some basil species. A previous study described the anti-inflammatory activity of the alcoholic extract of *Ocimum gratissimum* leaves in the formalin-induced paw edema test (Tanko *et al.*, 2008). Moreover, the crude alcoholic extract of *O. basilicum* L. inhibited proinflammatory mediators, such as nitric oxide (NO) and interleukin-1β (IL-1β) (Selvakkumar *et al.*, 2007). The essential oil of *O. micranthum* L. also exerted antinociceptive effects on acetic acid-induced writhing and in the formalin test in mice (Lino *et al.*, 2005; Tanko *et al.*, 2008).

The therapeutic properties of basil result from the biological activity of some of its constituents, such as linalool and 1,8-cineole (Zhou *et al.*, 2007a; Martinez-Velazquez *et al.*, 2011; Nogueira de Melo *et al.*, 2011). These active compounds have particular therapeutic properties. 1,8-Cineole has been shown to have anti-inflammatory properties in airway diseases in clinical studies (Juergens *et al.*, 2003; Zhou *et al.*, 2007a; Martinez-Velazquez *et al.*, 2011), and linalool inhibits paw edema induced by Complete Freund's Adjuvant (Batista *et al.*, 2010).

However, few studies have investigated the anti-inflammatory properties of *Ocimum americanum* L. species. The present study investigated the effects of *Ocimum americanum* L. essential oil (OEO) and its isolated compounds, linalool and 1,8-cineole, on paw edema and in an experimental model of zymosan-induced arthritis.

Materials and Methods

Plant Material and Extraction of Essential Oil

Fresh leaves of *Ocimum americanum* L. were collected in March and April 2010 from a rural property in the city of Maringá, Paraná, Brazil. The plant was identified and authenticated by Dr. Roberto Fontes Vieira, a taxonomist at Embrapa in Brasilia, Brazil.

A voucher specimen was deposited in the herbarium of the botanical department of the State University of Maringá (no. 11160). The OEO was extracted by conventional steam distillation using a Clevenger-type apparatus for 3 h at a temperature of 70°C. The essential oil was kept at 4°C in dark vials and then used in the tests.

Essential Oil Analysis

Gas chromatography-mass spectrometry. Gas chromatography (GC) analysis was performed with a Thermo Electron Focus GC model under the following conditions: DB-5 capillary column (30 m × 0.32 mm, 0.50 mm); column temperature, 60°C (1 min) to 180°C at 3°C/min; injector temperature, 220°C; detector temperature, 220°C; split ratio, 1:10; carrier gas, He; flow rate, 1.0 ml/min. An injection volume of 1 μl was diluted in acetone (1:10). The GC-mass spectrometry (MS) analysis was performed using a Quadrupole mass spectrometer (Thermo Electron, DSQ II model), operating at 70 V. Identification of the individual components was based on comparisons of their GC retention indices on apolar columns and comparisons with mass spectra of authentic standards purchased from Sigma-Aldrich (Adams, 2001).

Nuclear magnetic resonance. ^{1}H (300.06 MHz) and ^{13}C nuclear magnetic resonance (NMR; 75.45 MHz) spectra were recorded in a deuterated chloroform ($CDCl_3$) solution in a Mercury-300BB spectrometer, with δ (parts per million [ppm]) and spectra referred to $CDCl_3$ (δ 7.27 for ^{1}H and 77.00 for ^{13}C) as the internal standard.

Experimental Animals

Female Balb/c mice, weighing 22 ± 3 g, were provided by the Central Animal House of the State University of Maringá. The animals were housed at 22 ± 2°C under a 12 h/12 h light/dark cycle with free access to food and water. All of the protocols were approved by the Ethics Committee for Animal Experimentation of the State University of Maringa (CAEA/UEM 066/2010).

Experimental Model of Zymosan-Induced Arthritis

For the experimental induction of zymosan-induced arthritis, 200 μg/cavity of zymosan A (Sigma, St. Louis, MO, USA) in 10 μl sterile saline was prepared. Thirty minutes before zymosan injection, the mice were orally treated with vehicle (1% Tween 80 solution), OEO, linalool, or 1,8-cineole at doses of 50, 150, or 300 mg/kg, dissolved in vehicle. The right knee joints of the animals were then intra-articularly injected with zymosan, and the contralateral knee joint was injected with an equal volume of saline (i.e., negative control). The animals were then anesthetized and sacrificed. Six hours later, the number of migrated leukocytes was counted. Seven days later, histological analysis was performed to measure cytokine levels.

Leukocyte migration. The knee joint was exposed by surgical incision and washed twice with 5 μl of phosphate-buffered saline (PBS) that contained ethylenediaminetetraacetic acid

(EDTA) and was diluted to a final volume of 50 μl with PBS/EDTA to determine total cell counts. The total number of leukocytes, diluted in Turk's solution, was determined in a Neubauer chamber under a light microscope (Zeiss, Wetzlar, Germany). The results are expressed as the number of leukocytes per cavity.

Zymosan-Induced Paw Edema

Edema was induced in the right hind paw of the mice by a subplantar injection of a zymosan suspension (200 μg/paw, 20 μl/paw) in sterile saline (0.9%). The contralateral paw was injected with saline (i.e., negative control; Winter *et al.*, 1962). The OEO (150 mg/kg), linalool (300 mg/kg), 1,8-cineole (300 mg/kg), or vehicle (1% Tween 80 solution) was administered orally 30 min before zymosan administration in the hind paw. The degree of edema was measured immediately before and 6, 12, and 24 h after zymosan injection using mercury plethysmography (Ugo-Basile, Comerio, Italy) that was specially modified for small volumes. The difference between the two paw volumes, determined before and after injection of the edema-provoking agent, indicated the severity of edema, and was evaluated as the percentage difference between the paw volume at each time-point and basal paw volume.

Histological Analysis

The right knee joints were subsequently demineralized in a 10% formaldehyde solution for three days, and formaldehyde was then replaced with EDTA dissolved in saline (3 g EDTA/30 ml). The joints were stored in 70% ethanol at 4°C. The samples were then subjected to a dehydration process using an increasing series of ethanol concentrations (70, 80, 90, and 100 GL) diaphanized in xylol, and embedded in paraffin. The samples were serially sectioned (4 μm thickness) using a rotary microtome (Leica RM2245). All of the sections were stained with Harris' hematoxylin and eosin (H&E) and examined under a microscope (Olympus BX41; original magnification, 400×). The histological sections from each group were examined using a grading scale of 0–3, according to the proportion of infiltrated cells: 0 (infiltrated cells equivalent to normal), 1 (poorly infiltrated cells), 2 (moderately infiltrated cells), and 3 (densely infiltrated cells). Cartilage destruction was similarly graded on the same 0–3 scale, ranging from no damage to fully destroyed cartilage layers: 0 (cartilage equivalent to normal), 1 (cartilage destruction in one to two areas), 2 (cartilage destruction in three or more areas), and 3 (complete cartilage destruction). The semiquantitative scale was adapted from Weinberger *et al.* (2003) and Nishida *et al.* (2004).

Measurement of Cytokine Levels Using Enzyme-Linked Immunosorbent Assay

The knee joints were placed in 500 μl PBS that contained EDTA. The tissues were homogenized (homogenizer model D-130, Biosystems) and then centrifuged. The supernatant was separated, rapidly frozen, and stored at −70°C for the later analysis of interferon γ (INF-γ) and transforming growth factor β (TGF-β) using commercial enzyme-linked

immunosorbent assay (ELISA) kits. The protocol followed the manufacturer's recommendations (R&D Systems). Cytokine levels in the supernatant are expressed as picograms per milliliter.

Statistical Analysis

All of the data are expressed as mean ± S.E.M. The results were statistically analyzed using two-way analysis of variance (ANOVA) or one-way ANOVA followed the Tukey test. Values of $p < 0.05$ were considered statistically significant.

Results and Discussion

The chromatographic analysis of OEO resulted in the identification of 26 components, representing 98% of the total OEO. The results of the GC-MS analysis (Fig. 1) of the OEO components were characterized by high percentages of linalool (19.63%), 1,8-cineole (17.27%), eugenol (14.67%), and camphor (14.06%). The percentages of the major components and their retention indices are summarized in Table 1.

Several preclinical studies of alternative herbal therapies have been conducted (Venkatesha *et al.*, 2011) because side effects associated with the long-term use of nonsteroidal and steroidal anti-inflammatory drugs have been observed in clinical practice. Although animal models have inherent limitations, they have contributed to the basic understanding of joint disease and development of effective antiarthritic agents (Asquith *et al.*, 2009; Ratheesh *et al.*, 2009; Jung *et al.*, 2012). In the present study, OEO significantly reduced leukocyte migration in the knee joint and edema in the right hind paw induced by zymosan

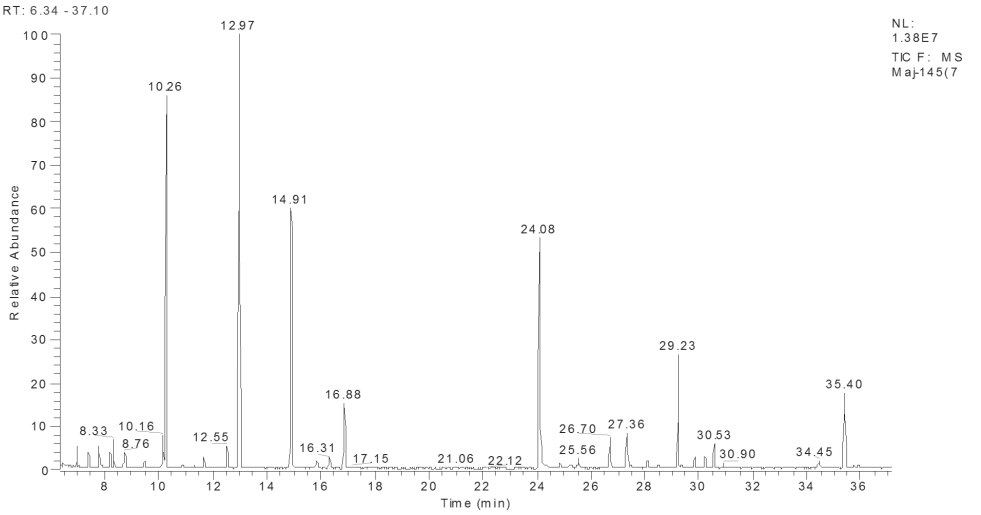


Figure 1. Chromatographic profile of the essential oil of *Ocimum americanum* L. Peak identification is reported in Table 1.

Table 1. Percent Chemical Composition of Essential oil of *Ocimum americanum* L.

Retention Time	Compound	Percent (%)*	Identification
6.96	α-pinene	1.04	GC/MS, NMR
7.43	Camphene	0.79	GC/MS, NMR
8.21	Sabinene	0.59	GC/MS, NMR
8.33	β-pinene	1.42	GC/MS, NMR
8.76	β-mircene	0.89	GC/MS, NMR
10.16	Limonene	1.26	GC/MS, NMR
10.26	1,8 cineole	17.27	GC/MS, NMR
11.67	linalool oxide	0.81	GC/MS
12.55	Fenchone	1.28	GC/MS
12.97	Linalool	19.63	GC/MS, NMR
14.91	Camphor	14.06	GC/MS, NMR
15.87	Borneol	0.76	GC/MS, NMR
16.31	4-terpineol	0.74	GC/MS, NMR
16.88	α-Terpineol	3.33	GC/MS, NMR
17.15	*cis*-piperitol	0.45	GC/MS
17.94	Estragole	0.08	GC/MS, NMR
24.08	Eugenol	14.67	GC/MS, NMR
24.87	β-selinene	0.36	GC/MS
25.56	—	0.85	No identified
26.70	β-caryophyllene	1.80	GC/MS, NMR
27.36	α-*trans*-bergamotene	2.01	GC/MS, NMR
28.09	α-humulene	0.61	GC/MS
29.23	germacrene D	6.89	GC/MS, NMR
29.84	biciclogermacrene	0.99	GC/MS
30.21	β-bisabolene	0.96	GC/MS, NMR
30.53	γ-muurolene	1.47	GC/MS, NMR
35.4	γ-cadinene	4.62	GC/MS, NMR
35.9	—	0.33	Not identified

Note: * Relative percentage of chemical constituents.

in Balb/c mice. The experimental model of zymosan-induced arthritis is associated with neutrophil recruitment, lymphocyte proliferation, synovial hypertrophy, and pannus formation, with biphasic arthritis that consists of both early (< day 7) and late (> day 25) phases (Frasnelli *et al.*, 2005; Asquith *et al.*, 2009; Pinho *et al.*, 2012). The OEO at doses of 150 and 300 mg/kg significantly inhibited leukocyte migration to the articular cavity in Balb/c mice (Fig. 2A).

The inflammatory response after an intraplantar injection of zymosan into the right hind paw showed a time-dependent response, with maximal swelling at 6–12 h that returned to baseline 96 h after zymosan injection (Fig. 2B). Similarly, a previous study found that paw inflammation in rats is maximal 3–4 h after zymosan injection, followed by a reduction of swelling at 72 h (Colucci *et al.*, 2008). Zymosan-induced paw inflammation in mice induces peripheral edema, cyclooxygenase 2 (COX-2) expression, primary hyperalgesia, and central sensitization (Jain *et al.*, 2008). In the present study, OEO at 150 mg/kg reduced

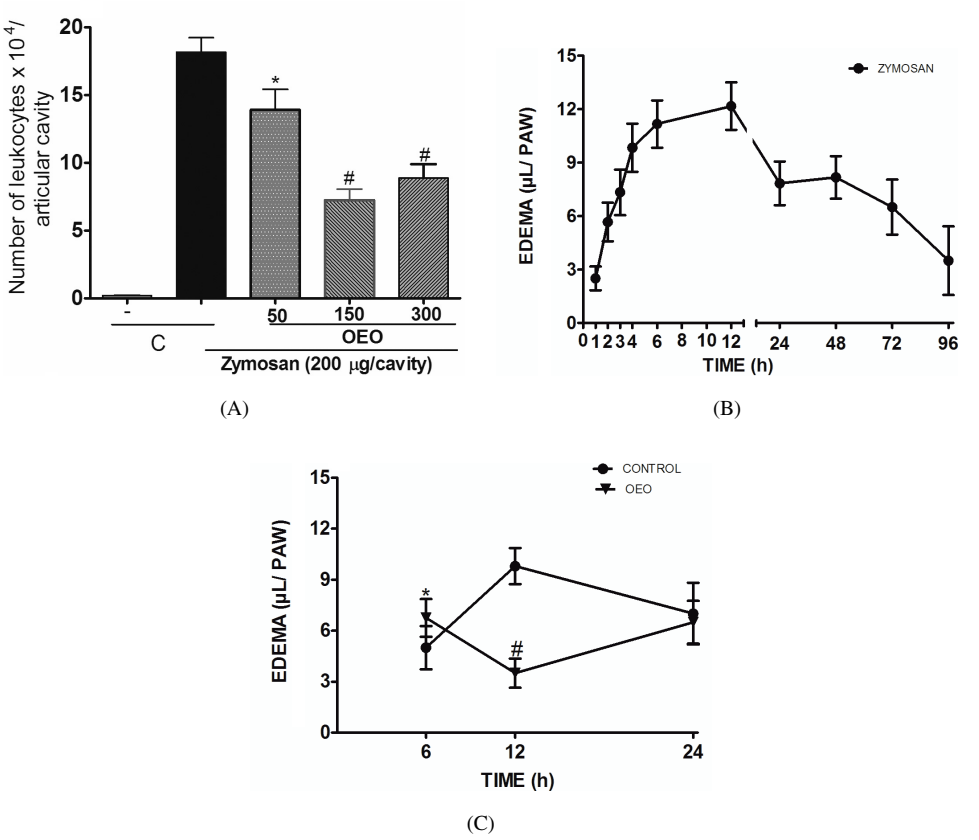


Figure 2. The OEO reduced leukocyte migration in zymosan-induced arthritis and paw edema. (A) The mice were orally treated with OEO (50, 150, or 300 mg/kg) or vehicle 30 min before zymosan challenge (200 μg/knee). Leukocyte migration was evaluated 6 h after intraplantar zymosan injection. (B) Paw edema was evaluated at the indicated times after zymosan injection (200 μg/mouse). (C) The mice were treated with OEO (150 mg/kg) or vehicle, and paw edema was evaluated at 6, 12, and 24 h. The data are expressed as the mean and SEM ($n = 5$ mice). $^{*}p < 0.05$, compared with vehicle; $^{\#}p < 0.05$, compared with zymosan challenge (ANOVA followed by Turkey test).

paw edema in mice, with a maximal effect at 12 h (Fig. 2C). This antiedematous effect suggests that the inhibitory action of OEO may be attributable to the suppression of the release of mediators responsible for inflammation, including prostaglandin E2 (PGE2) and COX-2 (Setty and Sigal, 2005).

Experimental arthritis promoted the accumulation of inflammatory cells in the synovial membrane and cartilage damage, indicated by the histological analysis (Fig. 3). Zymosan, which is a ligand for toll-like receptor 2 and activator of the alternative complement pathway, triggers local activation of the innate immune system, causing inflammation in the injected joint (Frasnelli *et al.*, 2005; Keystone *et al.*, 1977). At early time points after zymosan injection, edema formation is accompanied by neutrophil infiltration and the production of inflammatory mediators in the synovial tissue and fluid in the inflamed joints.

Later time points after zymosan injection are characterized by a chronic response, in which macrophage and lymphocyte accumulation occurs (Pettipher and Salter, 1996; Penido *et al.*, 2006). In the present study, the effect of zymosan injection was observed as an intense inflammatory reaction, characterized by increased leukocyte migration in the articular cavity.

An increase in polymorphonuclear cell infiltration in the synovial membrane and cartilage destruction were also observed (Figs. 3B_1, B_2, B_3) compared with control animals (Figs. 3A_1, A_2, A_3). Furthermore, we found that oral treatment with OEO (150 mg/kg) for seven days in arthritic animals inhibited leukocyte migration in the synovial membrane

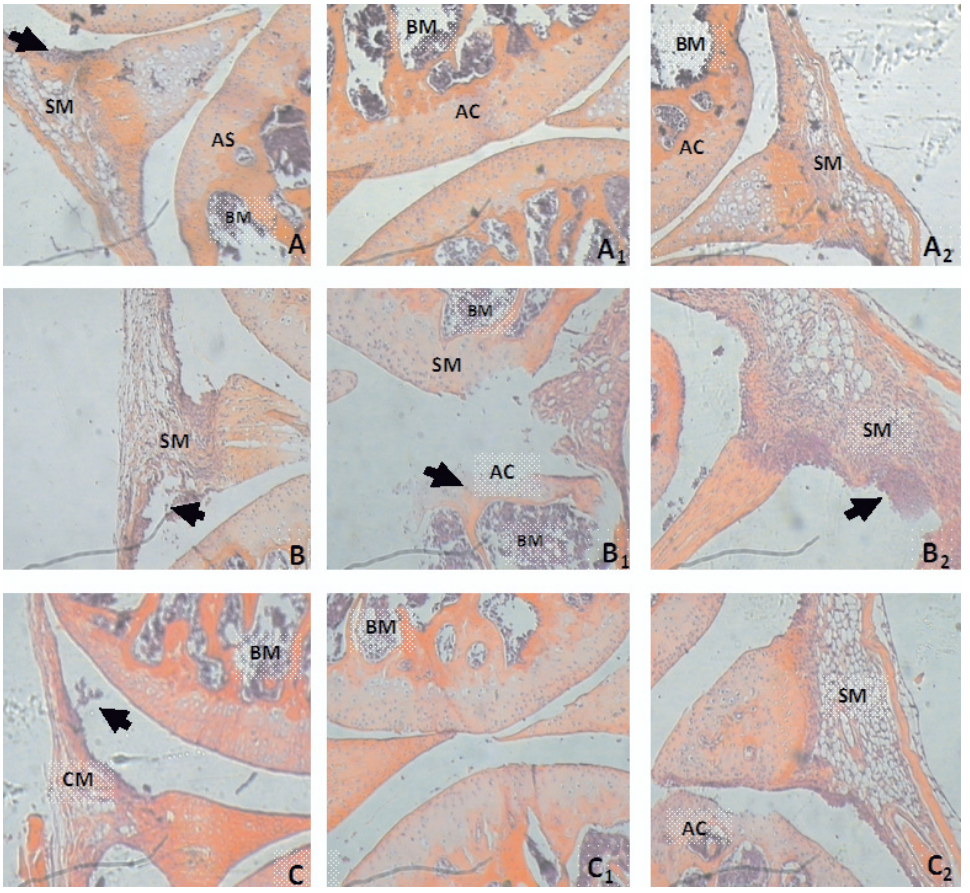


Figure 3. Histological analysis of OEO-treated mice in an experimental model of zymosan-induced arthritis. Areas of inflammatory cell infiltration and cartilage destruction were depicted and analyzed. (A_1, A_2, A_3) Knee joints from mice after challenge with vehicle (negative control). (B_1, B_2, B_3) Knee joints from mice with zymosan-induced arthritis. (C_1, C_2, C_3) Animals orally treated with OEO (150 mg/kg) 30 min before zymosan injection and twice daily for seven days. The joint sections were stained with H&E (original magnification, 400×). Black arrows show inflammatory cell infiltration and cartilage destruction. SM, synovial membrane; AC, synovial cartilage; BM, bone marrow.

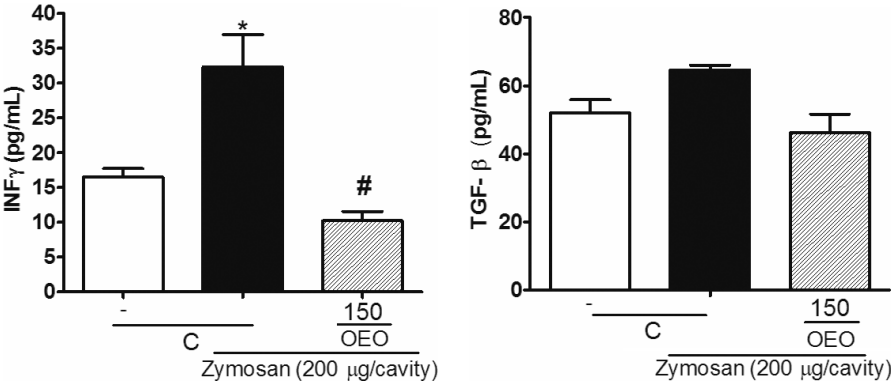
Table 2. Histological Grading Scale

Category	Control ($n = 5$)	ZYA ($n = 5$)	OEO ($n = 5$)	Score
Cell infiltrate				
Normal	4		2	0
Poorly infiltrated cells	1		3	1
Moderate infiltrated cells		3		3
Densely infiltrated cells		2		2
Cartilage destruction				
Normal	4		3	0
Poorly infiltrated cells	1	2	2	1
Moderate infiltrated cells		3		2
Densely infiltrated cells				3

Note: Modified from the scale described by Weinberger *et al.* (2003) and Nishida *et al.* (2004). Histological analysis of synovial membrane and cartilage damage were evaluated.

and attenuated cartilage destruction (Figs. $3C_1$, C_2, C_3) compared with the control group (Figs. $3B_1$, B_2, B_3). The histological grading scale is presented in the Table 2.

Cytokines, including TNF-α, IL-18, IL-1β, INF-γ, and IL-6, have been found in high concentrations in the synovium in rheumatoid arthritis patients and experimental models of arthritis and contribute to joint damage (Dai *et al.*, 2007; Pinto *et al.*, 2010; Rocha *et al.*, 2011). Herein, the cytokine levels of IFN-γ and TGF-β were determined in synovial tissue in mice pretreated with OEO and stimulated with zymosan (Fig. 4A). Although IFN-γ is present in the pathogenesis of arthritis, the mechanism in different models of arthritis is a subject of controversy (Rosloniec *et al.*, 2002; Lemos *et al.*, 2009). For example, in an experimental model of antigen-induced arthritis, neutrophil migration depends on prostaglandin, which



(A) (B)

Figure 4. Effect of OEO on INF-γ and TGF-α levels in knee joints in arthritic mice. Animals were orally treated with OEO (150 mg/kg) 30 min before zymosan injection and twice daily for seven days. Control mice were challenged with saline. The concentrations of (A) INF-γ and (B) TGF-α were determined by ELISA seven days after zymosan injection. $^{*}p < 0.05$, compared with control mice; $^{\#}p < 0.05$, compared with zymosan-injected mice (ANOVA followed by Tukey test).

enhances IL-17 synthesis and inhibits IL-12/IFN-γ production (Li *et al.*, 2010). However, oral treatment with an extract of *Impatiens pritzellii* Hook reduced IFN-γ levels in a model of collagen-induced arthritis (Zhou *et al.*, 2007b). In the present study, an intra-articular injection of zymosan significantly increased IFN-γ levels compared with control animals, and OEO treatment for seven days significantly reduced IFN-γ levels in synovial tissue (Fig. 4B). TGF-β exerts both pro- and anti-inflammatory effects. Its possible beneficial effects in rheumatoid arthritis include the inhibition of cartilage degradation, inhibition of lymphocyte proliferation, and suppression of macrophage superoxide production. TGF-β also has chemotactic properties at the site of inflammation and stimulates cells to produce proinflammatory cytokines, such as IL-1, IL-6, and TNF-α (Drynda *et al.*, 2002). Herein, OEO treatment did not interfere with TGF-β levels (Fig. 4B), suggesting that this essential oil does not appear to be involved in the mechanism of action of TGF-β.

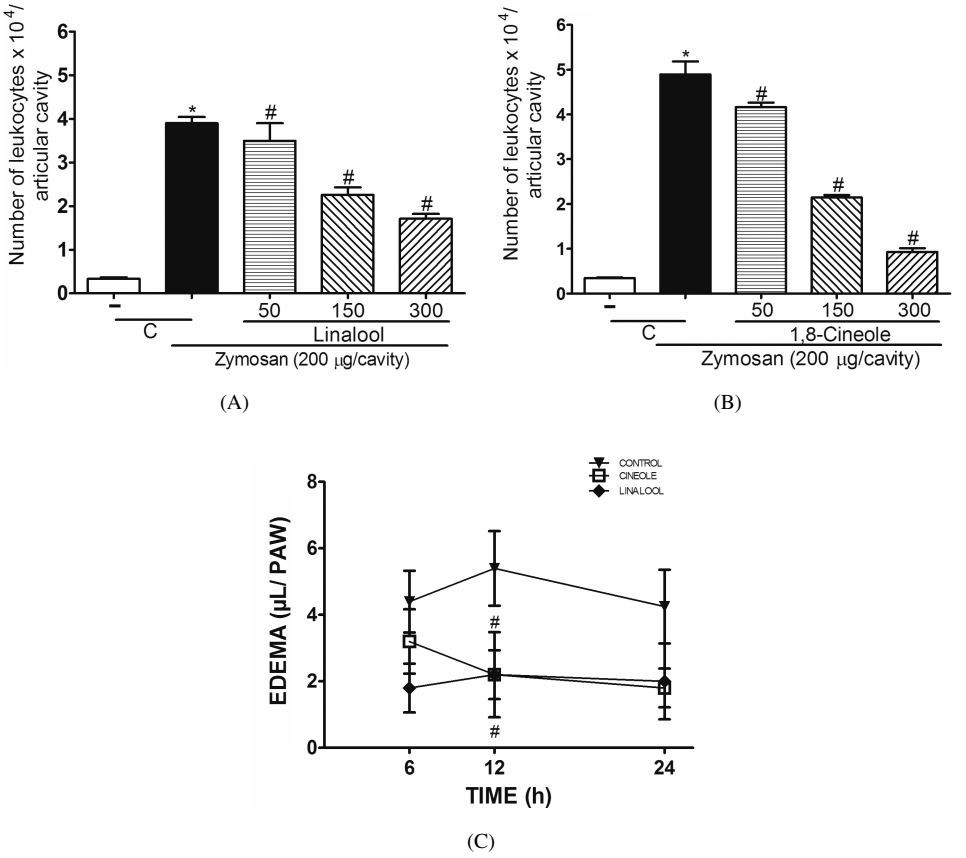


Figure 5. Effect of linalool and 1,8-cineole on leukocyte migration in an experimental model of zymosan-induced arthritis and paw edema in mice. The mice were orally treated with 50, 150, or 300 mg/kg (A) linalool or (B) 1,8-cineole 30 min before zymosan injection (200 μg/knee). (C) Paw edema was evaluated at the indicated times (6, 12, and 24 h) after zymosan injection (200 μg/paw). The data are expressed as mean and S.E.M. ($n = 5$ mice). *$p < 0.05$, compared with vehicle; $^{\#}p < 0.05$, compared with zymosan challenge (ANOVA followed by Tukey test).

The present results suggest a suppressive effect of this essential oil in zymosan-induced arthritis. Our next step was to investigate the effect of its major components, linalool and 1,8-cineole. Linalool (Fig. 5A) and 1,8-cineole (Fig. 5B) reduced leukocyte migration at all of the doses tested (50, 150, and 300 mg/kg), indicating that these compounds are important inhibitors of leucocyte migration. Linalool and 1,8-cineole also inhibited paw edema formation at 12 h (Fig. 5C). Previous studies have shown that linalool reduces thermal hyperalgesia induced by carrageenan and causes antinociception when assessed in a formalin-induced nociception model in mice (Peana *et al.*, 2003). 1,8-Cineole exerted anti-inflammatory effects in an experimental model of inflammation in rats (Santos and Rao, 2001; Santos *et al.*, 2004; Chao *et al.*, 2005). Juergens *et al.* (2003) studied patients with bronchial asthma and observed anti-inflammatory effects of 1,8-cineole. In conclusion, the present results suggest that OEO and its isolated components linalool and 1,8-cineole have anti-inflammatory effects, inhibiting leukocyte migration in zymosan-induced arthritis and reducing paw edema. Further studies are necessary to elucidate their mechanisms of action.

Acknowledgments

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